DRUG RESIDUES IN ANIMAL TISSUES

Screening Test for Sulfamethazine and Sulfathiazole in Swine Liver

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A screening method is described for estimation of sulfamethazine and sulfathiazole residues in swine livers. Tissues are homogenized with $CHCl_3$-ethyl acetate (1 + 1). The drugs are extracted from the organic solvent with pH 10 carbonate buffer and back-extracted into dichloromethane, as an ion-pair with tetrabutylammonium hydroxide, without pH adjustment. Following evaporation of the solvent, the residue is dissolved in methanol, subjected to thin layer chromatography, and detected by the Bratton-Marshall reaction. Recoveries of sulfamethazine and sulfathiazole, determined by high pressure liquid chromatography, were 50.8 and 42%, respectively, with coefficients of variation of 4.2 and 4.7%.

Sulfa drugs are widely used in animal production. In swine, sulfamethazine and, to a lesser degree, sulfathiazole, are the drugs of choice. Although federal regulations require animal withdrawal from drug use long enough to limit residues in swine livers to 0.1 ppm, the Food Safety and Inspection Service (FSIS), U.S. Department of Agriculture, reported (1) that 4.2% of the livers examined in 1980 were in violation of the tolerance level. The violation rate, although unacceptable, represents a significant decrease compared with the 15% rate determined in 1978 (2).

FSIS and the Food and Drug Administration have relied primarily on a modification of the Tishler et al. procedure (3) to detect and quantitate sulfa drug residues in swine livers. The limitations of this procedure with regard to time for analyses (5–7 samples/analyst/day) and reliability of results are well established (4). The economic advantage of a more rapid and reliable screening procedure, especially in view of the decrease in violations in the past 2 years, is evident.

To be totally effective, a laboratory screening procedure must be relatively rapid, capable of detecting drug residues at the violative level, free of false positive results, and must tentatively identify the contaminating drug and demonstrate reproducible results over a wide range of concentrations. The screening methods currently proposed for detecting sulfa drugs in swine tissues fail to meet one or more of these criteria. The procedure for detecting sulfamethazine and sulfathiazole in swine livers presented here was designed specifically to meet these requirements.

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Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

METHOD

Reagents

(a) *Solvents.*—Ethyl acetate and dichloromethane (DCM) (Distilled-in-Glass®, Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442); $CHCl_3$, "Baker Analyzed" reagent (J. T. Baker Chemical Co., Phillipsburg, NJ 08665).

(b) *Tetrabutylammonium hydroxide (TBAH).*—Aldrich Chemical Co., Inc., Milwaukee, WI 53233. 40% aqueous solution.

(c) *N-1-(Naphthyl)ethylenediamine (NEDA) dihydrochloride.*—Sigma Chemical Co., St. Louis, MO 63178. 0.4% methanol solution.

(d) *0.1M Carbonate buffer.*—pH 10. Prepare from 0.1M solutions of sodium carbonate and sodium bicarbonate.

(e) *Sulfamethazine.*—American Cyanamid Co., Princeton, NJ 08540.

(f) *Sulfathiazole.*—Sigma Chemical Co.

Apparatus

(a) *Tissue grinder.*—Brinkmann Polytron® homogenizer (Brinkmann Instruments Inc., Westbury, NY 11590).

(b) *Centrifuges.*—International clinical centrifuge-rotor No. 273 (International Equipment Co., Needham Heights, MA 02194); Sorvall superspeed centrifuge-type SS-1 rotor (Ivan Sorvall, Inc., Norwalk, CT).

(c) *High pressure liquid chromatographic (HPLC) system.*—Laboratory Data Control (Riviera Beach, FL 33404) Constametric pump controlled by gradient master programmer and connected to

Schoeffel Model SF770 Spectroflow monitor operated at 254 nm. Rheodyne 7120 sampling valve. Column, 30 cm × 4 mm id μBondapak C_{18}. Mobile phase, water–methanol–acetic acid (80 + 20 + 0.5) with 0.1% TBAH. Samples eluted isocratically at flow rate of 1 mL/min.

(d) *Vortex stirrer.*—Super mixer (Lab-Line Instruments, Inc., Melrose Park, IL 60160).

(e) *Thin layer chromatographic (TLC) system.*—2.5 × 10 cm glass plate with 250 μm layer of silica gel G (Analtech, Newark, DE 19711). Developing solvent, ethyl acetate–methanol (4 + 1).

Procedure

Weigh 2.5 g ground frozen liver (5) into 50 mL polypropylene centrifuge tube. Let liver partially thaw. Add 16 mL cooled (4.4–10°C) $CHCl_3$–ethyl acetate (1 + 1) and blend 30 s with Polytron homogenizer at low speed. Centrifuge 2 min at 3000 rpm. Remove solvent with disposable Pasteur pipet and filter through small plug of glass wool packed in disposable super Pasteur pipet, collecting filtrate (ca 12.5 mL) in 15 mL screw-cap centrifuge tube. Add 2 mL pH 10 buffer and shake carefully in rocking motion for 2 min. Centrifuge 2 min at 2500 rpm. Carefully transfer aqueous phase to a second 15 mL screw-cap centrifuge tube with aid of Pasteur pipet. Add 30 μL TBAH solution and vortex-mix 15 s. Add 13 mL DCM and shake vigorously 3 min. Centrifuge 2 min at 2500 rpm. Carefully pour contents of centrifuge tube into 60 mL separatory funnel containing 2 mL DCM in manner to prevent mixing of phases. Let stand 1 min. Slowly draw off DCM phase into 23 mL screw-cap specimen vial. Evaporate solvent in 9 mL screw-cap specimen vial at 50°C under stream of nitrogen, transferring entire solution with disposable Pasteur pipet.

Dissolve residue in 0.2 mL methanol and spot 10 μL in 1.0–1.5 μL increments, drying spot with aid of stream of nitrogen between applications. Maintain diameter of spot at ≤4 mm. Develop plates 0.5 cm from origin. Dry plates in 60°C forced air oven and redevelop plates to height of 3 cm from origin. Dry plates in forced air oven before spraying with visualizing reagents.

Modified Bratton-Marshall (BM) Color Development (6)

Expose plates for 5 s to nitrous acid vapors generated by addition of sodium nitrite to 8% aqueous phosphoric acid (use hood). Allow 10 s for excess nitrous acid to dissipate. Spray with NEDA reagent to produce pink spots.

Recovery Studies

Absolute drug recoveries by the screening procedure were determined by HPLC of liver extracts. Drug-free liver samples were spiked by injection into tissue with sulfamethazine and sulfathiazole at 0.10, 0.20, 0.44, and 0.60 ppm before analysis. In addition, extracts of drug-free livers were spiked with the various concentrations of the drugs to prepare standard curves. Drug-free liver extracts served as a control for both. Residues of liver extracts were dissolved in 0.08 mL methanol, and 0.32 mL water–acetic acid–TBAH (80 + 0.5 + 0.1) was added. Mixture was vortexed 30 s, followed by centrifugation for 1 min at 2500 rpm. Thirty microliter samples were injected onto the HPLC column. Recoveries were determined on the basis of peak heights. Retention times were 10.5 min for sulfamethazine, and 7.0 min for sulfathiazole.

Results and Discussion

The method described has been successfully applied to swine livers spiked with sulfamethazine and sulfathiazole, as well as livers containing naturally incurred sulfamethazine at or above the violative level. The use of ethyl acetate–methanol (4 + 1) as the TLC developing solvent, together with restricting the diameter of sample spot applied at the origin to 4 mm and solvent migration to 3 cm, results in compact bands (sulfamethazine, R_f 0.9; sulfathiazole, R_f 0.8) on the TLC plates. As a result, this enables the detection of 0.02 ppm of the drugs in liver.

The use of the pH 10 carbonate buffer for extracting the sulfa drugs from an organic solvent, rather than the use of an acid solution as in most proposed methods, serves 2 important purposes. First, it limits potentially interfering BM-positive aromatic amines (e.g., procaine) to amphoteric compounds. Second, and more important, by the technique of ion-pairing with TBAH (7), the sulfa drugs are back-extracted into an organic solvent without the need for pH adjustment of the aqueous solution.

Analyses of approximately 25 livers consistently resulted in the detection of an unknown BM-positive compound in varying concentrations. The unknown (R_f 0.6), characterized by its slow color development relative to sulfamethazine and sulfathiazole, does not, however, interfere with determining the presence or absence of the drugs. The identification of the unknown is currently under investigation.

The absolute percent recoveries of sulfa-

Table 1. Recovery of sulfathiazole and sulfamethazine added to swine liver

	Recovery,[a] %	
Added, ppm	Sulfathiazole	Sulfamethazine
0.1	44.0	50.5
0.1	45.8	50.8
0.1	40.5	50.1
0.1	40.9	46.5
0.2	41.0	47.7
0.2	43.2	49.7
0.2	39.6	53.8
0.2	44.4	55.1
0.44	40.7	52.1
0.44	38.5	50.1
0.44	41.3	51.2
0.44	42.9	48.9
0.6	42.6	54.1
0.6	39.6	51.0
0.6	43.7	50.8
0.6	43.1	50.8

[a] HPLC recovery, based on actual quantity of drug recovered relative to quantity of drug added.

methazine and sulfathiazole, as determined by HPLC, from 16 swine liver samples spiked with concentrations ranging from 0.1 to 0.6 ppm are presented in Table 1. The mean recoveries were 50.8 and 42.0%, respectively, with coefficients of variation of 4.2 and 4.7%. Because relatively consistent recoveries were obtained over a range of sulfa drug concentrations, an internal standard is not considered necessary in the procedure. Furthermore, the approximate concentration of the contaminating drug can be determined by comparing the color intensities of positive samples with that of appropriate drug standards. Analysts, therefore, can determine those positive samples which merit more lengthy and elaborate quantitation and confirmation studies.

In the method outlined, higher absolute recoveries of the sulfa drugs were sacrificed to minimize the time of analysis. In this respect, an individual analyst who performs 4 concurrent analyses can complete 20 samples in an 8-h period.

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